INTEGRITY OF CHICKEN RNA AS AFFECTED BY PROLONGED POSTMORTEM DURATION

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Abstract –This study aimed investigating stability of RNA from chicken skeletal muscle as a function of PM intervals representing the end-points of evisceration, carcass chilling and aging stages in chicken abattoir. Chicken breast muscle was dissected from the carcasses (n=6) immediately after evisceration, and one-third of each sample was instantly snap-frozen and labeled at 20-min PM. The remaining muscle was stored on ice until the next rounds of sample collection (1.5 h and 6 h PM). The delayed PM duration did not significantly affect A_{260}/A_{280} ($p \geq 0.05$). Slight decreases in intensity of 28s and 18s rRNA bands were observed, indicating a minor RNA degradation. However, total RNA was still intact up to 6 h. Transcript abundances of genes commonly used as references were determined using qPCR. Abundances of beta-actin, glyceraldehyde 3-phosphate dehydrogenase, and hypoxanthine-guanine were significantly different among all PM time points ($p<0.05$). The findings suggest an adverse effect of prolonged PM duration on reliability of transcript quantification in chicken skeletal muscle.

Key Words – Gene expression, RNA stability, Skeletal muscle.

I. INTRODUCTION

Accuracy of gene expression study in the animals considerably relies on high quality and integrity of mRNA isolated from the tissue of interest [1-4]. Animal tissue collected immediately after slaughter is the ideal sample providing high quality of RNA samples. As postmortem (PM) duration proceeds, RNA is progressively degraded unless immediately preserved by deep-freezing. However, this practice is difficult and inconvenient to execute in an actual industrial abattoir. Recent studies have shown that RNA isolated from skeletal muscle of human [5], pigs [6] and cattle [7] may be stable if the tissue samples have been handled appropriately. However, no investigation of RNA stability in chicken skeletal muscle has been documented. The objective of this study was to evaluate RNA quality and integrity in chicken breast meat as a function of time PM to establish the possible utility of aging PM chicken muscle in gene profiling experiments.

II. MATERIALS AND METHODS

Six Ross-308 broiler carcasses (2.4-2.7 kg) were purchased from a local abattoir (Nakhorn Pathom, Thailand). Sample collection was performed at the abattoir according to standard industry practice. Each chicken carcass was taken from the processing line immediately after evisceration (2 min PM). Skeletal muscle (*pectoralis major*) from one side of the breast was dissected from the carcass and the skin was removed. The sample was divided into 3 portions. The first portion was immediately diced into small cubes, snap frozen in liquid nitrogen. The remaining breast muscle from the same breast side was placed in a clean plastic bag and stored on ice until the next rounds of sample collection (1.5 h PM and 6 h PM). The time points at 1.5 h and 6 h represented the end-points of chilling and aging steps, respectively. All tubes were kept in liquid nitrogen while the samples were transferred back to BIOTEC and stored at -80°C until used for RNA isolation.

Isolation of RNA and integrity assessment

Total RNA was isolated from chicken muscle samples (100 mg frozen sample) using QIAzol® lysis reagent (Qiagen, Inc., Valencia, CA) according to the manufacturer's instruction. Quantity and purity of total RNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.). Integrity of total RNA was determined by agarose electrophoresis. High integrity of RNA was generally denoted by two clean bands of 28s and 18s rRNA on the agarose gel [8].

Quantitative real-time polymerase chain reaction

Capability of the isolated RNA to serve as the qPCR template was investigated. Total RNA (1.2 μg) was reverse transcribed into cDNA using iScript™ Select cDNA Synthesis Kit (Bio-Rad, CA) following the company's protocol. The oligo$(dT)_{20}$ was used as the primer for the amplification. The qPCR of common reference genes (beta-actin, *ACTB*; glyceraldehyde 3-phosphate dehydrogenase, *GAPDH;* hypoxanthine-guanine phosphoribosyltransferase, *HPRT*; peptidylprolylisomerase A *PPIA*; TATA box-binding protein, *TBP*) was performed. Primers of chicken genes (Table 1) were designed using Beacon Designer™ software (PREMIER Biosoft, Palo Alto, CA). The qPCR reactions included 25 ng cDNA, 1 μM primer mix, and 10 μL of SsoAdvanced™ Universal SYBR® Green supermix (Bio-Rad) according to the manufacturer's instruction. The reaction was performed in a Real Time PCR model BIORAD CFX96 (Bio-Rad). Specificity of PCR products was confirmed by melting curve analysis. Threshold cycle (Ct) was analyzed using Bio-Rad CFX Manager 2.1 software (Bio-Rad). Expression at 1.5 h or 6 h relative to 20 min PM was calculated using $2^{-\Delta Ct'}$ method, where $\Delta Ct' = Ct_{1.5h \text{ or } 6h} - Ct_{20 \text{ min}}$ Different letters above the bars indicate significant difference ($p<0.05$) in expression of each gene at different time points. [9].

Statistical analysis

Statistical analysis was conducted using the SPSS program, version 11.5 (SPSS Institute Inc., Cary, NC). The groups of data with significant difference in mean ($p<0.05$) were subjected to Duncan's new multiple range test.

Table 1 Primers for quantitative real-time polymerase chain reaction

Gene ID	Sequence[1] 5'→ 3'	Amp[2] (bp)	T_m[3] (°C)
ACTB	**F:** CAAAGCCAACAGAGAGAAG	137	83.5
	R: CATCACCAGAGTCCATCA		
GAPDH	**F:** ACTTTGGCATTGTGGAGGGT	131	86.0
	R: GGACGCTGGGATGATGTTCT		
HPRT	**F:** CTCACAGGCTTCAACTCT	115	80.0
	R: AATGACCAAGACGAGATTCT		
PPIA	**F:** TCTCAACATCTCACATCTCT	136	88.5
	R: AAGCAGCACTTAACTAATTGT		
TBP	**F:** TGCAATCACCAAAGGTCACCA	87	81.5
	R: TGCAGTAGCTGAAGCTGTGTT		

[1] F = forward, R = reverse
[2] Amp = amplicon length
[3] T_m = melting temperature

III. RESULTS AND DISCUSSION

Quantity and purity of the total RNA isolated from chicken skeletal muscle obtained at different PM interval were determined by UV spectrophotometry (Figure 1). The average A_{260}/A_{280} of 1.83±0.11 indicates an absence of protein contamination in the isolated RNA. No significant differences in A_{260}/A_{280} were found among the tissues collected at different time points ($p\geq0.05$).

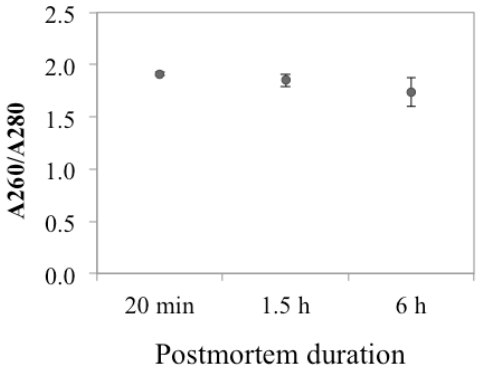

Figure 1. Relative absorbance at 260 nm to 280 nm (A_{260}/A_{280}) of chicken breast muscle collected at different postmortem time-points.

The effect of PM time on integrity of total RNA was assessed by agarose gel electrophoresis. Based on the electrophoretic profiles (Figure 2),

28s and 18s rRNA bands of 20 min PM samples exhibited the greatest intensity compared to samples of 1.5 h and 6 h PM. The electrophoretic patterns suggested degradation of rRNA at the extent PM duration. However, at 6 h PM, two bands of 28s and 18s rRNA population were still clearly illustrated, indicating intact RNA.

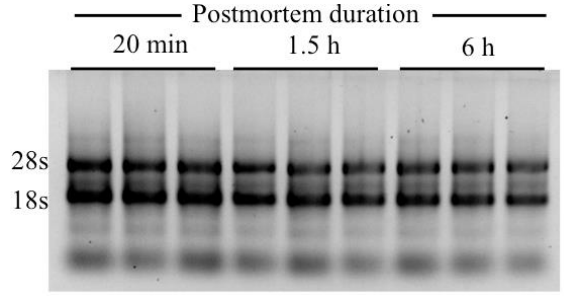

Figure 2. Electrophoretic profiles of total RNA from chicken skeletal muscle obtained at different postmortem periods.

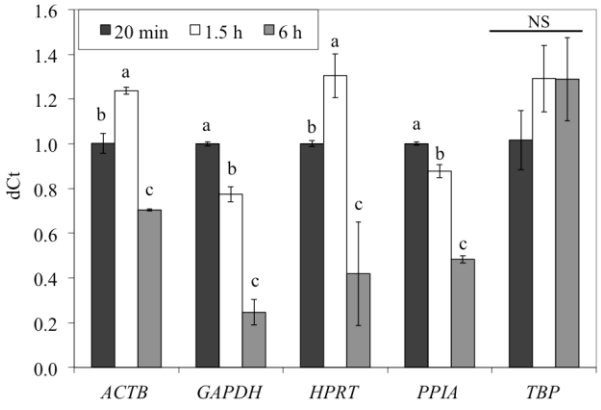

Figure 3. Transcript abundance of five reference genes in chicken skeletal muscle as postmortem extended. Bars represent fold changes (mean±SEM) in expression of genes at 1.5 h or 6 h postmortem relative to 20 min PM. Relative expression of one gene was calculated using $2^{-\Delta Ct'}$ method, and illustrated as dCt. NS = no significance ($p \geq 0.05$).

To evaluate the effect of delayed PM time on mRNA stability, the relative abundance of the selected genes was analyzed using qPCR. Relative abundance describes change in abundance of the target genes in 1.5-h or 6-h PM samples relative to 20-min PM group. Of five selected reference genes (Figure 3), abundance of *ACTB* and *HPRT* was slightly higher at 1.5 h PM ($p<0.05$), but significantly decreased at 6 h PM ($p<0.05$). For *GAPDH* and *PPIA*, abundance of those transcripts continuously decreased as PM duration extended ($p<0.05$). Conversely, no significant difference in *TBP* abundance was found among all PM time points ($p \geq 0.05$). The results indicated a decrease in mRNA levels of four out of five selected reference genes in the chicken skeletal muscle collected at 6 h PM.

As total RNA of each time point, for each biological replicate, was isolated from the same piece of chicken skeletal muscle, the reduced transcript abundance implied an impaired integrity of mRNA potentially due to endogenous nucleases catalysis. However, within 6 h PM, the catalysis might not remarkably reduce quantity of the intact rRNA population as two sharp bands 28s and 18s rRNA were still observed.

It is apparent that impact of delayed PM duration depends on PM metabolic rate. Since PM metabolism of bovine and swine skeletal muscle exhibit slower rate compared to chicken [10], RNA degradation was slower in cattle and pigs. In cattle, PCR products of *GAPDH* and *ACTB* were reported in skeletal muscle stored at 4°C up to 22 days [7], while in pigs, degradation of RNA isolated from skeletal muscle appeared at 48h PM [8].

The initial intention of this study was to test the possibility of using chicken muscle collected after carcass chilling or aging steps in transcript quantification, as sample collection after evisceration step was inconvenient and wasteful. Although, abundance of *TBP* remained unchanged after 6 h PM, most of the reference genes exhibited altered mRNA abundance at 1.5 h PM. Still, the chicken muscle collected at 20 min showed the best quality of RNA.

IV. CONCLUSION

Total RNA isolated from chicken skeletal muscle was stable up to 6 h PM. However, transcript abundance of reference genes, including *ACTB*, *GAPDH*, *HPRT* and *PPIA* genes significantly changed when RNA was isolated from the muscle stored on ice for 1.5 h and 6 h. The findings suggested an adverse impact of an extent PM

period on integrity of mRNA isolated from chicken skeletal muscle. To preserve the best quality of RNA aimed for subsequent gene expression study, particularly transcriptome analysis, chicken skeletal muscle should be immediately collected after evisceration or within 20 min PM. It is recommended that if prolonged PM chicken muscle tissue must be used in such investigation, a preliminary experiment should be conducted to inspect the effect of the delayed sample collection on mRNA stability of gene of interest.

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